

Skin resident memory CD8+ T cells are phenotypically and functionally distinct from circulating populations and lack immediate cytotoxic function.

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Summary

The in-depth understanding of skin resident memory CD8⁺ T lymphocytes (TRM) may help to uncover strategies for their manipulation during disease. We investigated isolated TRM from healthy human skin, which expressed the residence marker CD69, and compared them to circulating CD8⁺ T cell populations from the same donors. There were significantly increased proportions of CD8⁺CD45RA[–]CD27[–] T cells in the skin that expressed low levels of killer cell lectin-like receptor G1 (KLRG1), CD57, perforin and granzyme B. The CD8⁺ TRM in skin were therefore phenotypically distinct from circulating CD8⁺CD45RA[–]CD27[–] T cells that expressed high levels of all these molecules. Nevertheless, the activation of CD8⁺ TRM with T cell receptor (TCR)/CD28 or interleukin (IL)-2 or IL-15 in vitro induced the expression of granzyme B. Blocking signalling through the inhibitory receptor programmed cell death 1 (PD)-1 further boosted granzyme B expression. A unique feature of some CD8⁺ TRM cells was their ability to secrete high levels of tumour necrosis factor (TNF)- α and IL-2, a cytokine combination that was not seen frequently in circulating CD8⁺ T cells. The cutaneous CD8⁺ TRM are therefore diverse, and appear to be phenotypically and functionally distinct from circulating cells. Indeed, the surface receptors used to distinguish differentiation stages of blood T cells cannot be applied to T cells in the skin. Furthermore, the function of cutaneous TRM appears to be stringently controlled by environmental signals in situ.

Introduction

T cells are abundant in healthy human adult skin and are an important component of the local immune system ^{1,2}. Tissue resident T cells (TRM) arise from naive precursors that are first activated in secondary lymphoid organs and then migrate to the peripheral tissues, where they are retained through expression of CD69 and may act as sentinels for future reinfection ³⁻⁵. Current literature suggests that TRM may have constitutive functional activity and are poised to respond rapidly to antigenic challenge ^{1,2}. However, the mechanisms controlling the effector functions of these cells, particularly their cytotoxic function, are not known.

There has been extensive characterization of surface markers that can be used to identify circulating T cells at different stages of differentiation ^{6,7}. The correlation between phenotype and function has enabled the discrimination between naive (TN; CD45RA⁺CD27⁺), central memory (TCM; CD45RA⁺CD27⁺), effector memory (TEM; CD45RA⁻CD27⁻) and effector memory T cells that re-express CD45RA (TEMRA; CD45RA⁺CD27⁻) within both CD4⁺ and CD8⁺ T cell subsets ^{7,8}. In peripheral blood, highly differentiated TEM and TEMRA cells display high constitutive levels of preloaded cytotoxic granules containing perforin and granzyme B and can secrete the effector cytokines tumour necrosis factor (TNF)- α and interferon (IFN)- γ after activation in vitro ⁷. In addition these cells have short telomeres ⁹⁻¹¹, lack the co-stimulatory receptors CD27 and CD28, cannot produce the cytokine interleukin (IL)-2 but express high levels of CD57 and the inhibitory receptor killer cell lectin-like receptor G1 (KLRG1) ^{7,11,12}. These circulating cells also express high levels of the transcription factors T-bet and Eomesodermin (Eomes), that are important for cytotoxic differentiation ¹³. Overall, TEM and TEMRA are thought to have gained increased effector capabilities at the expense of proliferative activity compared to TN and TCM cells ^{7,11,12}. Unlike TN and TCM cells, the TEM and TEMRA populations do not home to lymphoid tissues, but may migrate instead to peripheral organs, including the skin ^{14,15}. Because most TRM in the skin lack the lymph node homing receptor C-C chemokine receptor type 7 (CCR7), they were thought initially to belong to the same lineage as TEM in the blood ^{1,16}. However, the mechanisms that control the activity of skin TRM in situ and their phenotype are poorly defined.

The aim of this study was to investigate the cytotoxic potential of CD8⁺ TRM in the skin of healthy donors and to identify the factors that influence the function of these cells. We found that CD8⁺ TRM are not poised for immediate cytotoxic activity, as they contain very low levels of perforin and granzyme B relative to effector CD8⁺ T cells in the blood. CD8⁺ TRM cells require cytokine- or T cell receptor (TCR)-mediated activation in order to adopt a cytotoxic effector phenotype, while inhibitory signalling through programmed cell death 1 (PD-1) may prevent this. In addition, phenotypical markers that identify highly differentiated cells in the blood (KLRG1, CD57, CD28⁻) are not characteristics of EM (CD27⁻CD45RA⁻) or EMRA (CD27⁻CD45RA⁺) TRM cells in the skin. Therefore, the correlation of differentiation stage of CD8⁺ T cell subsets with surface phenotype, which has been used widely to characterize circulating cells, cannot be extrapolated to the study of TRM populations in the skin. Instead, the skin appears to be populated with phenotypically distinct and diverse quiescent T cells, including a novel CD8⁺ T cell subset capable of producing both IL-2 and TNF- α upon stimulation.

Material and methods

Blood and skin samples

Blood, skin and blister samples were obtained from healthy donors or individuals undergoing plastic surgery. Individuals suffering from co-morbidities or on medication were excluded from the study. We investigated 33 skin donors in total (average age: 46 years, age range = 19–82, four males, 29 females). Written and informed consent was obtained from all participants, in agreement with the declaration of Helsinki protocols, and the study was approved by the Ethics Committee of the Royal Free Hospital and Guy's Hospital, London.

Sample preparation

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK). Cutaneous single-cell suspensions were obtained by overnight digestion of finely minced skin specimen with 0.8 mg/ml collagenase type IV (Life Technologies, Paisley, UK) in RPMI. Addition of 20% fetal calf serum (FCS) to the digestion mix prevented surface receptor degradation¹⁷. The cells from digested skin showed negligible surface marker degradation and similar phenotypical properties to those analysed in snap-frozen skin sections in situ via immunofluorescence, as reported previously¹⁸. Skin cells were obtained as described previously from suction blisters raised on normal skin¹⁹.

Flow cytometry

Antibodies used for flow cytometry are summarized in Supporting information, Table S1. Single-cell suspensions were stained initially for extracellular markers, followed by intracellular staining according to the manufacturers' instructions using either the Fix & Perm Cell Permeabilization Kit (An Der Grub, Buckingham, UK) for cytokines, perforin and granzyme B or intranuclear forkhead box protein 3 (FoxP3) Staining Buffer Set (Miltenyi Biotec, Bisley, UK) for T-bet and Eomes staining. To assess cytokine production, cells were stimulated with 25 ng/ml phorbol myristate acetate (PMA) and 500 ng/ml ionomycin for 1 h, followed by addition of 5 µg/ml brefeldin A (Sigma-Aldrich, Poole, UK) and incubation for a further 5 h. Unstimulated cells were used as the negative control. Telomere

lengths were measured via flow cytometry using the flow-fluorescence in-situ hybridization (FISH) method, as described previously 9. Pooled samples of blood and carboxyfluorescein succinimidyl ester (CFSE)-stained blister cells were used for the flow-FISH method in order to ensure equivalent staining between both cell populations. All samples were acquired using LSRII or Fortessa flow cytometers (both from BD Biosciences, San Jose, CA, USA) and analysed using FlowJo (TreeStar Inc., Ashland, OR, USA).

Immunofluorescence

Punch biopsies (5 mm diameter) from normal skin were frozen in optimal cutting temperature compound (OCT; Bright Instrument Company Ltd, Luton, UK); 6 μ m sections were cut, left to dry overnight, then fixed in ethanol and acetone and stored at -80°C . Samples were incubated with optimal dilutions of primary antibodies followed by an appropriate secondary antibody conjugated to various fluorochromes, as described 18. Antibodies used were: CD69 (clone FN50; Biolegend, San Diego, CA, UK), CD103 (clone 2G5.1; Thermo Fisher Scientific, Waltham, MA, USA), CD8 (clone RPTA-T8; BD Bioscience, San Jose, CA, USA), CD4 (YNB46.1.8; BD Bioscience) and PD-1 (clone NAT105; Abcam, Cambridge, UK). Images were acquired on the AxioScan Z1 slide scanner and Imaged on Zen Blue (Zeiss, Cambridge, UK).

Culture conditions

Cells were maintained in RPMI with 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine (all by Sigma-Aldrich). PBMCs or cells derived from collagenase digested skin were incubated for 4 days in the presence of no stimulant, 10 ng/ml TNF- α , 5 ng/ml IL-2 (both PeproTech, London, UK), 10 ng/ml IL-15 (R&D Systems, Abingdon, UK) or anti-CD3/CD28 coated beads (Dynabeads; Life Technologies, Paisley, UK), followed by flow cytometric staining. For PD-1 signalling blockade, cells were incubated with 10 mg/ml of anti-PD-1 ligand (PDL)-1 and anti-PDL-2 antibodies (MIH1 and MIH18) or 20 mg/ml isotype control (all eBioscience, Sa Diego, CA, USA) for 2 h prior to stimulation with 0.05 μ g/ml immobilized anti-CD3 (OKT3) for 3 days.

Statistical analysis

Graphs were drawn and statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Non-parametric data were identified using the D'Agostino and Pearson omnibus normality test. Statistical tests used included the Student's t-test or paired t-test when data followed a Gaussian distribution and the Mann–Whitney U-test or Wilcoxon's test for non-parametric data. Correlations were calculated using Pearson's correlation coefficient or Spearman's correlation for non-parametric data. Lines of best fit were generated using linear regression. If more than two groups were compared simultaneously, one-way analysis of variance (anova) (for parametric data) or the Friedman test (for non-parametric data) were applied, followed by Holm–Sidak or Dunn's multiple comparison tests, respectively, for paired comparisons. Differences were considered significant when $P < 0.05$.

Results

Skin-derived CD8⁺ T_{RM} cells express CD69 and are enriched for CD27⁻CD45RA⁻ (effector memory-like) populations

Single-cell suspensions were generated through overnight collagenase digestion of fresh healthy human skin and CD8⁺ T cell phenotype was analysed at the same time as PBMCs from the same healthy donors (representative gating strategy in Fig. 1a). The majority of skin but not blood-derived CD8⁺ T cells expressed CD69 that identifies them as tissue resident T cells ²⁰ (Fig. 1b–d). In the blood, combined use of the surface markers CD27 and CD45RA allows the identification of CD8⁺ T cells that have functional characteristics of naive (CD45RA⁺CD27⁺), TCM (CD45RA⁻CD27⁺), TEM (CD45RA⁻CD27⁻) and late stage/senescent TEMRA (CD45RA⁺CD27⁻) cells ^{7,21}. Among skin CD8⁺ T cells, the relative expression of CD45RA and CD27 identified four subsets as found in blood (Fig. 1e,f). However, skin CD8⁺ T cells were enriched significantly for the CD45RA⁻CD27⁻ T subset, while the CD45RA⁺CD27⁺ subset was reduced significantly in this tissue (Fig. 1e,f). Among the skin CD8⁺ T cells, CD69 expression was equally high in all four subsets (Fig. 1g). Similar changes were also observed in CD4⁺ T cells, where skin cells were mainly CD69⁺ and were enriched for CD45RA⁻CD27⁻ T cells compared to the blood of the same donors (Supporting information, Fig. S1a,b).

Skin CD8⁺ T_{RM} cells exhibit distinct expression of differentiation-associated surface markers compared to blood T cells

We next investigated whether skin CD8⁺ T_{RM} cells had the same phenotypical properties as their circulating counterparts. Circulating CD8⁺CD45RA⁻CD27⁻ and CD8⁺CD45RA⁺CD27⁻ T cells have been shown to express low levels of CD28 but high levels of the natural killer (NK) cell-associated receptors KLRG1 and CD57 compared to less differentiated populations ^{11, 22, 23}. Total skin CD8⁺ T_{RM} expressed similar levels of CD28 and CD57 but significantly lower levels of KLRG1 compared to the total circulating CD8⁺ T cell population (Fig. 2). However, when comparing the two tissues' CD8⁺CD45RA⁺CD27⁻ subsets, CD28 expression was significantly higher while CD57 and KLRG1 expression were significantly lower in the skin (Fig. 2). In addition, the CD8⁺CD45RA⁺CD27⁺ T_{RM} cells in the skin expressed significantly lower levels of CD28 and higher levels of CD57 than this

subset in the blood. Similar patterns were observed in skin CD4⁺ T cells, where the dominant CD45RA⁻CD27⁺ population did not express KLRG1 (Supporting information, Fig. 1). These results suggest that patterns of differentiation differ between blood and skin T cells.

To test the possibility that the CD8⁺ T cells in the skin and the circulation have disparate expression of differentiation-related markers because of differences in their replicative history, we compared the telomere lengths of these cells. In peripheral blood, CD8⁺CD45RA⁺CD27⁺ T cells have the longest and CD8⁺CD45RA⁻CD27⁻ T cells the shortest telomeres among T memory cell subsets ²⁴. As debris present in digested skin samples interfered with telomere detection, we isolated CD8⁺ T_{RM} cells by suction blister technology ²⁵ and compared them to circulating populations from the same donors (representative gating strategy shown in Supporting information, Fig. 2). Naive T cells had longer telomeres than CD45RA⁻CD27⁻ (T_{EM}) and CD45RA⁺CD27⁻ T (T_{EMRA}) subsets in both skin and blood CD8⁺ T cells (Fig. 2). Furthermore, telomere length of blood and skin total CD8⁺ T cells and there were similar (8.9 ± 1.8 kbp in the skin and 9.7 ± 2 kbp in blood CD8⁺ T cells; Fig. 2d). This indicated that the altered expression of differentiation-related markers between CD8⁺ T_{RM} and their blood counterparts was not due to differences in replicative history. However, telomere length was reduced significantly in skin compared to blood in total CD4⁺ T cells (8.0 ± 1.0 kbp in the skin *versus* 10.3 ± 1.8 kbp in the blood, Supporting information, Fig. 2b), indicating that skin CD4⁺ T_{RM} cells have experienced more proliferation *in vivo*.

A unique population of skin CD8⁺ T_{RM} secretes IL-2 and TNF- α after activation *in vitro*

Cutaneous CD8⁺ T_{RM} are considered a first line of defence against reinfection by pathogens ^{2, 26}. We investigated differences in cytokine production between isolated CD8⁺ T_{RM} and circulating CD8⁺ T cells. The production of three cytokines, IL-2, TNF- α and IFN- γ , individually or in combination, was measured after stimulation with PMA and ionomycin *in vitro* (Fig. 3). Among peripheral blood T cells, different subsets secrete either IL-2 alone or TNF- α together with IFN- γ ²³. In the skin, we observed a reduction in cells producing IL-2 alone and an increase in novel populations of CD8⁺ T cells, which lacked IFN- γ but secreted either TNF- α alone or in combination with IL-2 (Fig. 3b). CD8⁺ T_{RM} that

secreted both IL-2 and TNF- α were not confined to one particular CD45RA/CD27 population (Fig. 3c).

The average abundance of CD8⁺ T cells that secreted all three cytokines together after activation was not significantly different between blood and skin samples (Fig. 3b). However, slight but not significant differences were noted in the distribution of these multiple cytokine producers among the CD45RA⁺CD27⁻ subsets (Fig. 3d). These data indicate that the functional profiles of CD8⁺ T cells and their CD45RA/CD27 subsets are distinct in the blood and skin, and that the skin may contain unique populations of cells with different functional properties.

CD8⁺ T_{RM} in normal skin have low cytotoxic potential

CD8⁺ T_{RM} cells are considered to be poised for rapid responses in case of pathogen re-encounter ²⁶. Previous work from our group has shown that the T_{EM} and T_{EMRA} T cell subsets in the blood contain preloaded cytotoxic granules containing perforin and granzyme B ^{11,23}. Here, we show that the levels of both proteins were consistently lower in skin CD8⁺ T cells compared to the same subsets in blood, with perforin being almost undetectable in most individuals (Fig. 4). Furthermore, perforin and granzyme B levels were low in the CD8⁺CD45RA⁻CD27⁻ and CD8⁺CD45RA⁺CD27⁻ T cell subsets in the skin while being particularly high in the equivalent populations in the blood (Fig. 4c).

We next investigated whether the low cytotoxic mediator expression was induced locally in the skin or whether this was predetermined in T cells with skin homing potential. To do this we examined perforin and granzyme B expression in circulating T cells expressing the cutaneous lymphocyte antigen (CLA; Fig. 4d). Blood-derived CD8⁺CLA⁺ T cells expressed significantly less perforin and granzyme B than the CLA⁻ population (Fig. 4d), suggesting that cutaneous T cells lack cytotoxic effector functions prior to populating the skin.

Among skin-derived T cells, true resident cells express both CD103 and CD69 while cells transitioning in the tissue do not express either surface receptor ²⁰. We found that among skin CD8⁺

T cells granzyme B expression was highest in the minor 'transitioning' CD69⁻CD103⁻ population and lowest in the 'true resident' CD69⁺CD103⁺ population and that perforin was low in all these subsets (Fig. 4e). Furthermore, the transcription factors T-bet and Eomes, which are associated with effector memory T cell populations¹³, were both absent among CD8⁺ T_{RM} cells, even among those that were granzyme B-positive (Fig. 4f). Therefore, unlike circulating CD8⁺ T cells, CD8⁺ T_{RM} in healthy skin do not have a conventional effector phenotype and these cells lack immediate cytotoxic potential.

Surface markers that correlate with cytotoxic potential in circulating CD8⁺ T cells are not applicable in the skin

In the published literature, blood effector memory T cells are identified by their effector functions together with the increased expression of surface markers such as KLRG1 and the absence of other receptors, such as CD27 or CD28⁷. We confirmed a strong positive correlation between KLRG1 expression and granzyme B expression (Fig. 5a) and a negative correlation between both CD27 (Fig. 5b) and CD28 (Fig. 5c) with granzyme B expression in blood CD8⁺ T cells. This was not observed in cutaneous CD8⁺ T_{RM} cells. There is therefore a dissociation between the expression of this cluster of phenotypical markers and effector function of skin CD8⁺ T_{RM} cells, indicating further that memory CD8⁺ T cells from the circulation and the skin are controlled differently.

Cutaneous CD8⁺ T_{RM} skin T cells can be induced to express perforin and granzyme *in vitro*

Circulating CD8⁺ T cells can up-regulate perforin and granzyme B after stimulation^{27, 28}. In order to investigate whether skin-derived CD8⁺ T cells also have the ability to acquire cytotoxic potential, we activated total T_{RM} and PBMCs *in vitro* with or without either TNF- α , IL-2, IL-15 or anti-CD3/CD28 antibody-coated beads. After 4 days, perforin and granzyme B expression were measured via intracellular flow cytometry (Fig. 6a). All these stimulants, apart from TNF- α , induced a significant increase in granzyme B in both blood and skin CD8⁺ T cell populations (Fig. 6a). Perforin levels were increased significantly in both blood and skin CD8⁺ T cells by IL-15 or CD3/CD28 stimulation (Fig. 6a). Skin CD8⁺ T_{RM} cells are therefore capable of up-regulating their cytotoxic machinery to the same extent as blood populations, suggesting that these cells are not inherently unresponsive.

PD-1 signalling may be involved in low cytotoxic potential of skin T cells

PD-1 is a regulatory surface receptor often implicated in peripheral tolerance, and we hypothesized that PD-1 signalling may control granzyme B and/or perforin expression in skin T_{RM} cells. PD-1 levels were low among circulating CD8⁺ T cells but significantly higher in skin CD8⁺ T_{RM} cells (Fig. 6). PD-1 staining of T_{RM} was confirmed by immunohistology (Fig. 6d). To test whether PD-1 signalling affects perforin and granzyme B expression, healthy skin-derived cells were stimulated with suboptimal levels of plate-bound anti-CD3 antibody. This was performed in the presence of PD-L1 and PD-L2 blocking antibodies or isotype controls. In the presence of PD-1 ligand blockade, intracellular perforin was only increased significantly in blood-derived CD8⁺ T cells but not in skin-derived cells, while granzyme B was increased significantly compared to the isotype control in CD8⁺ T cells from both tissues (Fig. 6e). Therefore, while PD-1 signalling may contribute partially to the inhibition of cytotoxic potential of CD8⁺ T_{RM} in the skin, other mechanisms are also likely to be involved.

Discussion

A major advance in the understanding of immune memory was the recognition that memory T cells in tissues are an important barrier to reinfection and reactivation of pathogens ²⁶. Circulating T cells and T_{RM} cells have different transcriptional profiles, suggesting that their activity is differentially controlled ^{4, 5}. However, the mechanisms involved have not been defined. The aim of the present study was to compare the functional activity and extent of phenotypical differentiation of skin CD8⁺ T cells to those in the blood in healthy humans.

Blood-derived T cell subsets with distinct differentiation and functional properties are often identified through well-defined surface markers such as CD27 and CD45RA ⁷. We found that almost all skin-derived CD8⁺ T cells expressed the tissue retention marker CD69, identifying them as T_{RM} ²⁹. Among them, those that were CD45RA⁻CD27⁻ and CD45RA⁺CD27⁻ displayed low levels of perforin and granzyme B, substantially reduced expression of KLRG1 and CD57 and lacked the transcription factors T-bet and Eomes, but secreted both IL-2 and TNF- α after activation. This is clearly distinct from equivalent phenotypically defined CD8⁺ T cells in the circulation that have high perforin, granzyme, KLRG1, CD57, Eomes and T-bet and secrete TNF- α and IFN- γ , but no IL-2. Other studies using mRNA as a readout also report low levels of perforin, granzyme B or Eomes mRNA in healthy skin of humans or mice ^{5, 30}. This indicates that the correlation between effector phenotype and function that is observed in blood CD8⁺ T cells cannot be extrapolated to CD8⁺ T_{RM} populations, and a similar situation exists for CD4⁺ T_{RM} populations. Stated simply, cutaneous CD45RA⁺CD27⁺ CD8⁺ T_{RM} are not naive and CD8⁺CD45RA⁻CD27⁻ TRM cells are not immediately operational effector-like cells, unlike their blood counterparts.

Circulating cells expressing the skin homing marker CLA also lacked perforin and granzyme B, suggesting that the low cytotoxic potential of T_{RM} is pre-programmed in CD8⁺ T cells with a propensity for homing to the skin. Instead, we found that some cutaneous CD8⁺ T_{RM} cells can up-regulate their cytotoxic machinery after activation. Furthermore, cytotoxic T cells can be found in inflamed skin in patients with atopic dermatitis, psoriasis, herpes simplex-2 infection and vitiligo ^{2, 31, 32}. This suggests

that some T cells can become cytotoxic within the skin under appropriate stimulatory conditions *in vivo* and is in line with a recent report showing that among stimulated epidermal T_{RM}, one specific subset of CD49a⁺ cells is able to up-regulate cytotoxic effector molecules ³². This, together with our observation that T_{RM} share few phenotypical characteristics with highly differentiated cells in the blood, also supports previous murine work showing that skin T_{RM} can extensively proliferate locally and are derived from less differentiated circulating KLRG1⁻ memory precursor T cells ^{5, 33}.

We observed that the inhibitory receptor PD-1 was significantly higher on skin T_{RM} cells compared to CD8⁺ T cells in the circulation. Subsequent blockade of PD-1 signalling during TCR stimulation *in vitro* resulted in increased granzyme B but not perforin expression in the skin CD8⁺ T_{RM} cells, suggesting that other mechanisms may be involved in the repression of cytotoxic granule expression in skin CD8 T_{RM}. Granzyme B can cause extracellular matrix degradation ³⁴ and PD-1 signalling may therefore be involved in limiting CD8⁺ T cell-mediated tissue damage. PD-1 is reportedly elevated on brain, gastric mucosa and liver T cells ³⁵⁻³⁷, suggesting that PD-1 signalling may also be important in limiting the activity of resident CD8⁺ T cells in other tissues.

Cytokines produced by activated skin T_{RM} may also influence local immune responses. Large clusters of infiltrating T cells associated with a cytotoxic gene signature appear rapidly in human skin during challenge with varicella-zoster virus (VZV) antigens after vaccination against herpes zoster ^{38, 39}. We suggest that, after activation, a subset of CD8⁺ T_{RM}, with their unique cytokine secreting profile (IL-2⁺TNF- α ⁺), may activate endothelial cells through TNF- α ⁴⁰, thereby promoting the recruitment of cells from the circulation. Further IL-2 secretion by these cells would promote the expansion of the recruited cells at the site of challenge in the skin. Given that T cells expressing the skin-homing receptor CLA did not express perforin or granzyme B readily either, it is likely that recruited cells also may require further local stimulation to convert to a cytotoxic phenotype. Indeed, the importance of local effector T cell licensing in the skin was demonstrated in a murine graft-*versus*-host model, where T cell infiltration into the skin graft was not sufficient for rejection but required local Langerhans-mediated help for T cell cytotoxicity towards the mismatched graft ⁴¹.

Although skin resident CD8⁺ T_{RM} uniformly expressed CD69 and lacked perforin, our data also indicate heterogeneity among these cells in terms of cytokine production and expression of surface receptors such as CD27, CD28 CD45RA, KLRG1 and CD57. This suggests that similarly to blood cells, skin CD8⁺ T cells are composed of phenotypically distinct subsets. Indeed, differences between dermal and epidermal skin T cells have been reported previously²⁰. Epidermal CD8⁺ T cells in turn could be subdivided further into CD49a⁺ and CD49⁻ cells, which had the ability to produce cytotoxic effector molecules or IL-17, respectively³². We show that skin resident T cells cannot simply be grouped into subsets using surface marker expression patterns that have been established using blood cells. Full identification, characterization and compartmentalization of all phenotypical and functional subsets of the skin T cells therefore remains outstanding.

In summary, skin T_{RM} CD8⁺ T cells have low cytotoxic potential unless they are stimulated either by cytokines such as IL-15 or through the TCR. This, together with the negative regulation through PD-1, may be a mechanism that limits non-specific tissue damage under homeostatic conditions. Importantly, skin CD8⁺ T cells are phenotypically and functionally distinct from circulating T cells and must not be categorized based on blood-based surface markers, such as CD27 and CD45RA alone. The unique T_{RM} subset diversity, including IL-2/TNF- α -producers, will require further exploration with this in mind.

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Disclosures

The authors state no conflicts of interest.

Author contributions

J. A. S., S. M. H. and A. N. A. designed the study. J. A. S., M. V. S. and A. N. A. wrote the paper. B. M. D. performed the telomere measurements, N. P. the histological stains and J. A. S. the remaining experiments. J. F. D. provided technical expertise for histological analysis of skin samples. J. G. K. advised on the analysis of skin samples and the interpretation of the results. M. H. A. R. advised on recruitment of subjects and edited the manuscript. F. O. N. provided samples for analysis and advised on the initial outline of the project. K. E. L. provided access to the samples and edited the manuscript.

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Figure Legends.

Figure 1. Ex-vivo CD69 expression and CD27/CD45RA differentiation subset distribution in CD8⁺ T cells derived from the blood and skin. Collagenase digested skin and peripheral blood mononuclear cells (PBMCs) were analysed via flow cytometry. (a) Representative gating strategy used to identify CD8⁺ T cells in both tissues derived from the same donor. (b) Representative histograms and (c) cumulative data showing expression of the tissue residence marker CD69 and CD103 ($n = 8$). (d) Immunofluorescence staining for CD8, CD69 and CD103 in a human skin section. (e) Representative flow cytometry staining for CD27 and CD45RA and (f) cumulative CD27/CD45RA subset distribution in blood and skin derived cells ($n = 12$). (G) CD69 expression by CD27/CD45RA subset in blood and skin cells. T_{CM} = central memory, T_{EM} = effector memory and T_{EMRA} = effector memory re-expressing CD45RA.

Figure 2. Differentiation-associated phenotypical properties of skin compared to blood CD8⁺ T cells. (a) Representative and (b) cumulative *ex-vivo* measurements of CD28 ($n = 6$), receptor killer cell lectin like receptor G1 (KLRG1) ($n = 9$), and CD57 ($n = 8$), in CD8⁺ T cells and (c) in CD27/CD45RA subsets, measured via flow cytometry in collagenase digested skin and peripheral blood mononuclear cell (PBMC) samples derived from the same healthy donors. (d) KLRG1 expression among CD69⁻ and CD69⁺ skin cells ($n = 19$). (e) Cumulative data for total CD8⁺ T cells and T cell subsets for telomere length, measured via flow cytometry using the flow-fluorescence *in-situ* hybridization (FISH) method on PBMCs and skin blister-derived cells as described in the Material and methods section ($n = 5$).

Figure 3. Interleukin (IL)-2, interferon (IFN)- γ and tumour necrosis factor (TNF)- α cytokine production in blood and skin-derived CD8⁺ T cells. Collagenase digested skin cells or peripheral blood mononuclear cells (PBMCs) were stimulated *in vitro* with phorbol myristate acetate (PMA)/ionomycin before intracellular flow cytometry was performed to detect cytokine production ($n = 9$). (a) Representative dot-plots for IFN- γ and TNF- α staining. Histograms show IL-2 production by different subsets based on IFN- γ and TNF- α production; (b) cumulative data for total CD8⁺ T cells

producing IL-2, IFN- γ or TNF- α alone or in combination. Cytokine production by different CD8 subsets for (c) IL-2/TNF- α or (d) IL-2/IFN- γ /TNF- α cytokine co-production.

Figure 4. Ex-vivo expression of cytotoxic granule components in skin compared to blood CD8⁺ T cells. (a) Representative flow cytometry histograms and (b) cumulative data showing granzyme B and perforin expression in collagenase digested skin and blood derived CD8⁺ T cells from paired samples ($n = 9$). (c) Granzyme B and perforin expression among the CD8⁺ T cell subsets derived from blood ($n = 33$) and skin samples ($n = 16$). (D) Granzyme B and perforin expression in circulating CD8⁺ T cells positive or negative for the skin homing receptor cutaneous lymphocyte antigen (CLA) ($n = 12$). (e) Granzyme B and perforin expression in CD8⁺CD69/CD103 subsets in the skin of healthy donors ($n = 3$). (f) Expression of the transcription factors t-bet or Eomes with granzyme B in skin and blood CD8⁺ T cells.

Figure 5. Predictive value of CD27, CD28 and receptor killer cell lectin like receptor G1 (KLRG1) surface expression for intracellular granzyme B in skin and blood CD8⁺ T cells. Representative dot plots and cumulative data showing (a) KLRG1, (b) CD27 and (c) CD28 expression in relation to granzyme B expression in blood and skin-derived CD8⁺ T cells. Graphs show the correlations between percentage CD27, CD28 or KLRG1 expression and percentage granzyme B expression (lines of best fit, r -values and significance are shown where applicable). Further, cumulative data of granzyme B expression among CD27⁻/CD27⁺, CD28⁻/CD28⁺ and KLRG1⁺/KLRG1⁻ (with averages and standard errors) are shown. KLRG1 was measured in 18 blood and nine skin donors; CD27 and CD28 were measured in 33 blood and 17 skin donors.

Figure 6. Factors influencing granzyme B and perforin expression in blood and skin-derived CD8⁺ T cells. Skin and blood-derived cells from healthy donors were cultured for 4 days in the presence of tumour necrosis factor (TNF)- α , interleukin (IL)-2, IL-15 or anti-CD3/CD28, followed by intracellular flow cytometric staining. (a) Granzyme B and perforin expression [mean and standard error (s.e.)] in blood ($n = 12$) and skin-derived cells ($n = 10$). (b) Representative and (c) cumulative

data showing programmed cell death 1 (PD-1) expression measured by flow cytometry. (d) Immunofluorescent staining of healthy skin for CD8, PD-1 and nuclei [4',6-diamidino-2-phenylindole (DAPI)]. (e) Granzyme B and perforin expression following stimulation for 3 days with anti-CD3 in the presence of programmed cell death ligand 1/2 (PDL-1/2) blocking antibodies or isotype controls in blood ($n = 8$) and skin ($n = 6$) specimen [mean and standard error (s.e.) and P -values are shown].

Figure 1.

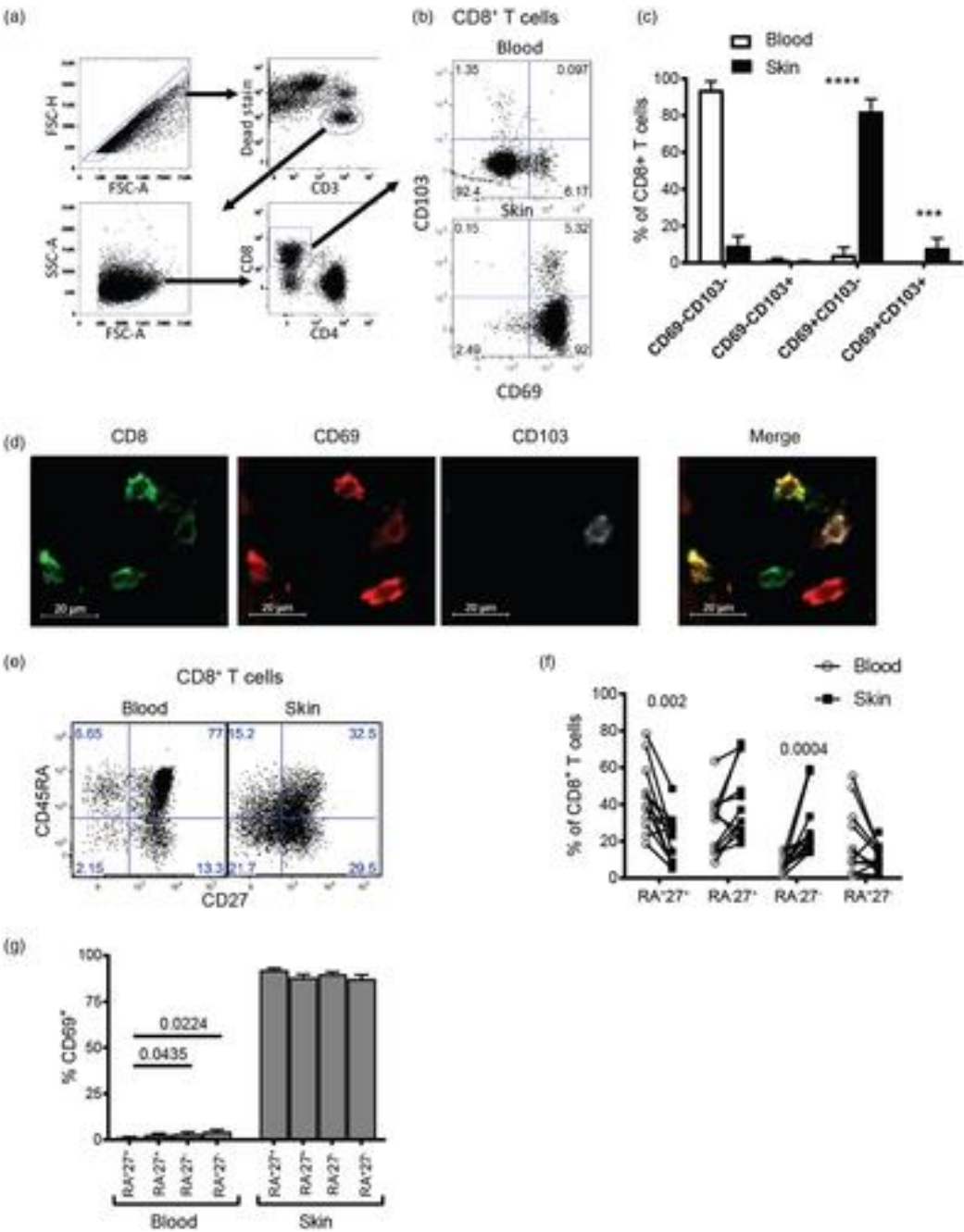


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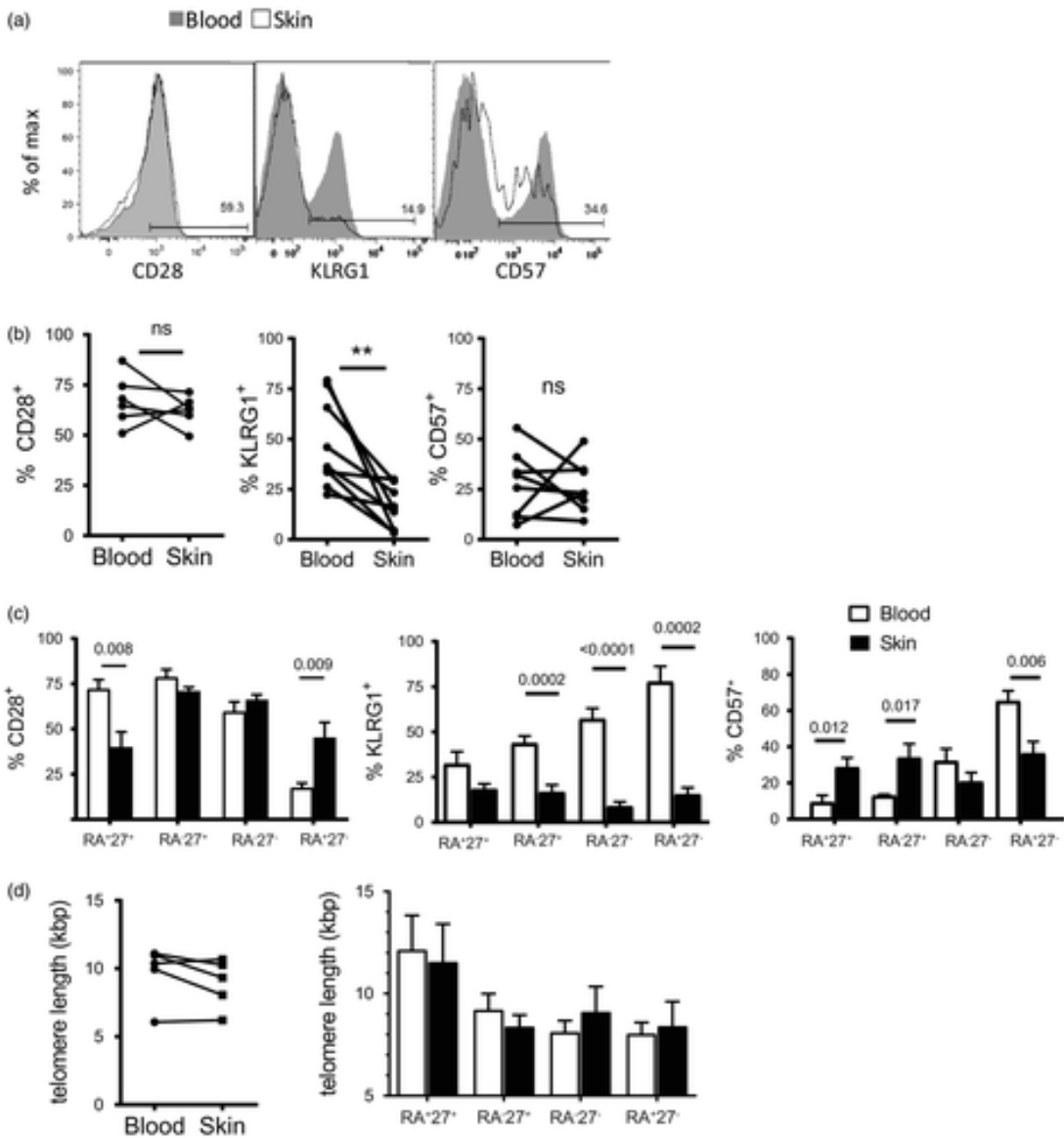


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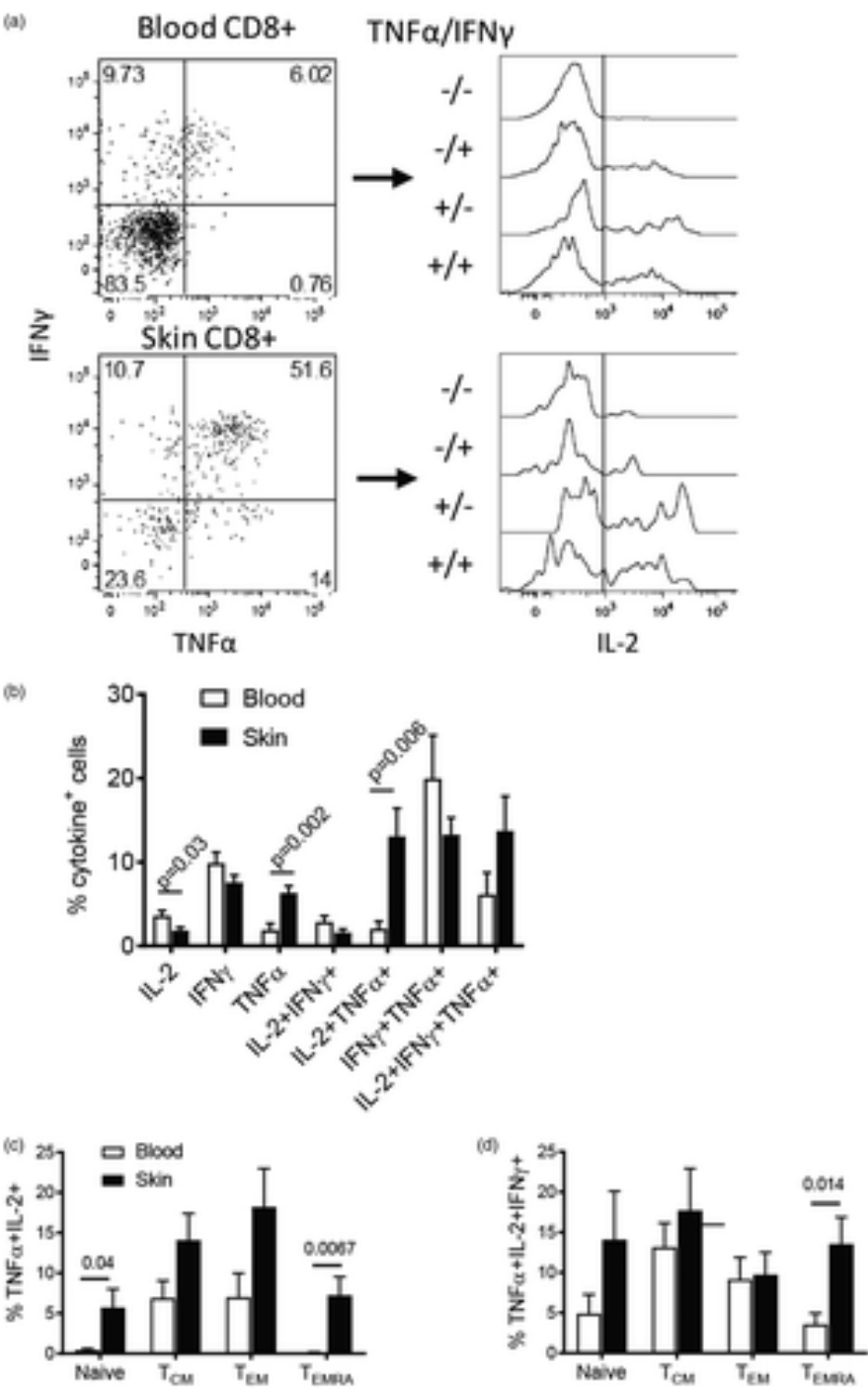


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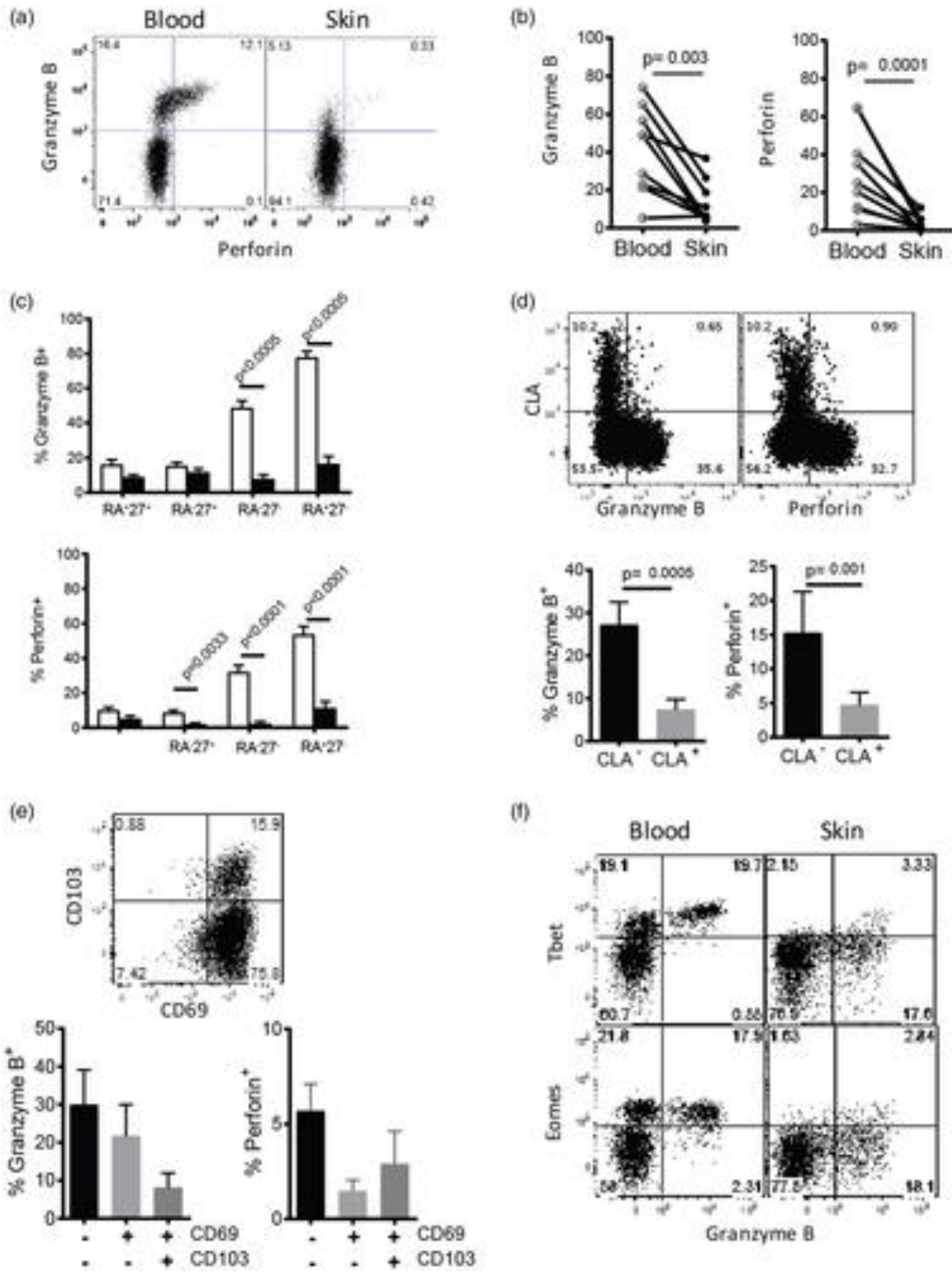


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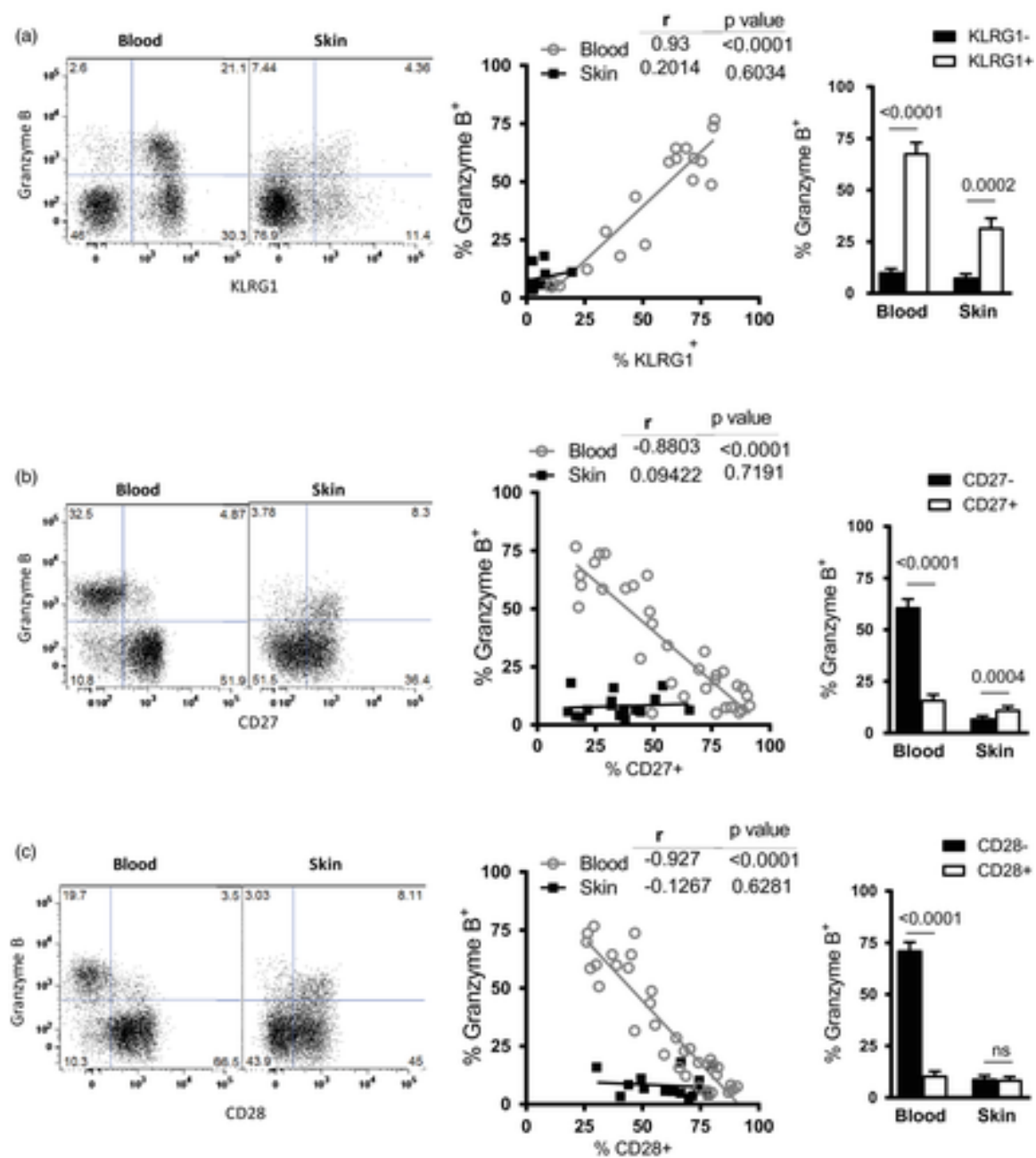
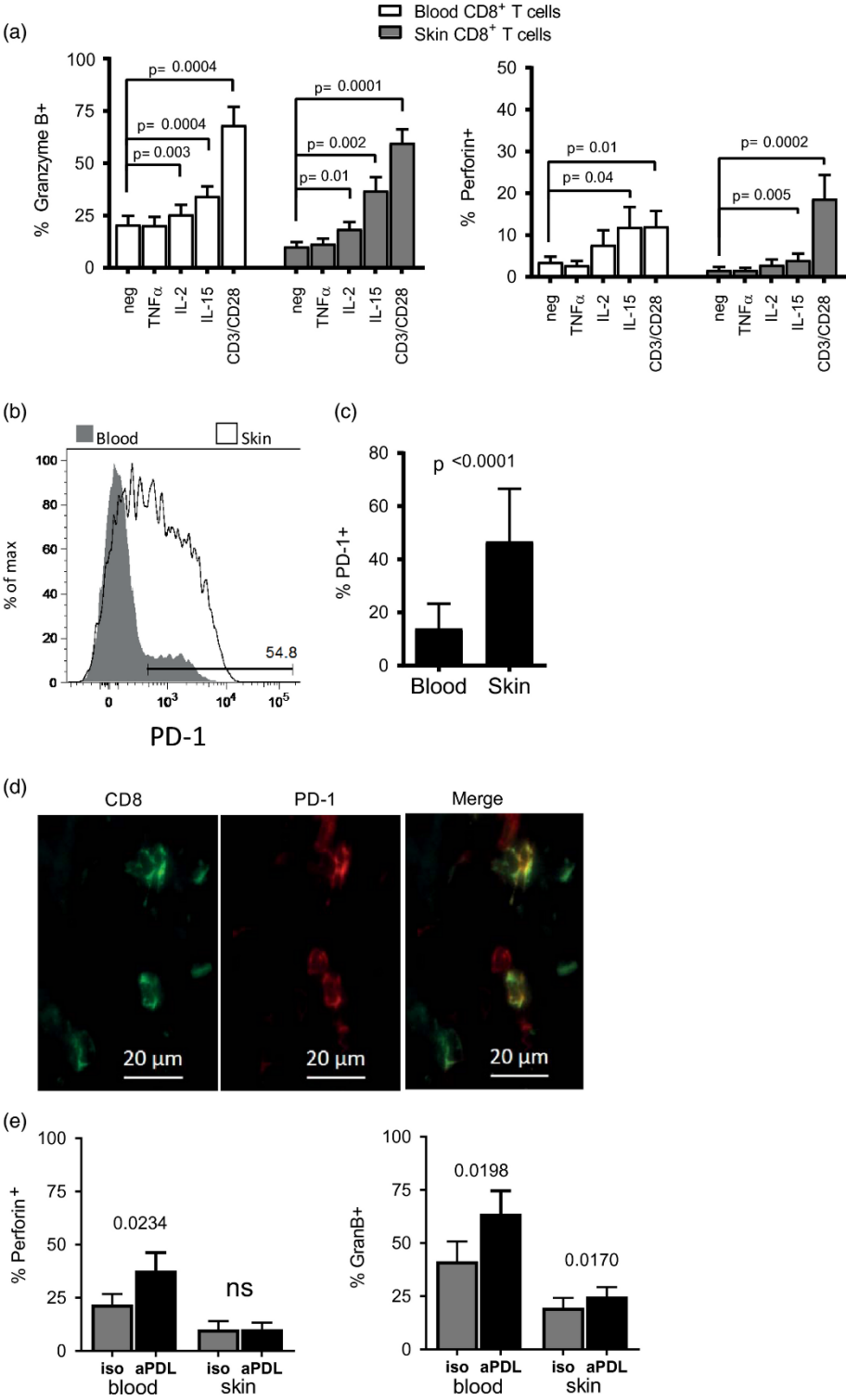


Figure 6.



Supporting figure legends.

Figure S1: Ex-vivo CD69 expression and CD27/CD45RA differentiation subset distribution in CD4⁺ T cells derived from the blood and skin. Collagenase digested skin and PBMCs were analyzed via flow cytometry. (a) Representative flow cytometry staining and (b) cumulative CD27/CD45RA subset distribution in blood and skin derived cells CD4⁺ T cells (n = 12). Expression of CD28 (n = 6), KLRG1 (n = 9) and CD57 (n = 8) among total blood and skin CD4⁺ T cells (c) and their subsets (d). (e) Skin cells or PBMCs were stimulated in vitro with PMA/Ionomycin before intracellular flow cytometry was performed to detect cytokine production. Cumulative data for total CD8⁺ T cells producing IL-2, TNF α or IFN γ alone or in combination.

Figure S2: Telomere length of skin and blood-derived CD4⁺ T cells. (a) Representative gating strategy for blood and skin CD4⁺ and CD8⁺ T cell subset telomere length measurements. (b) Cumulative data for total CD4⁺ T cells and CD27/CD45RA subsets for telomere length, measured via flow cytometry using the Flow-FISH method on PBMCs and skin blister derived cells as described in the material and methods section (n = 5).

Figure S1.

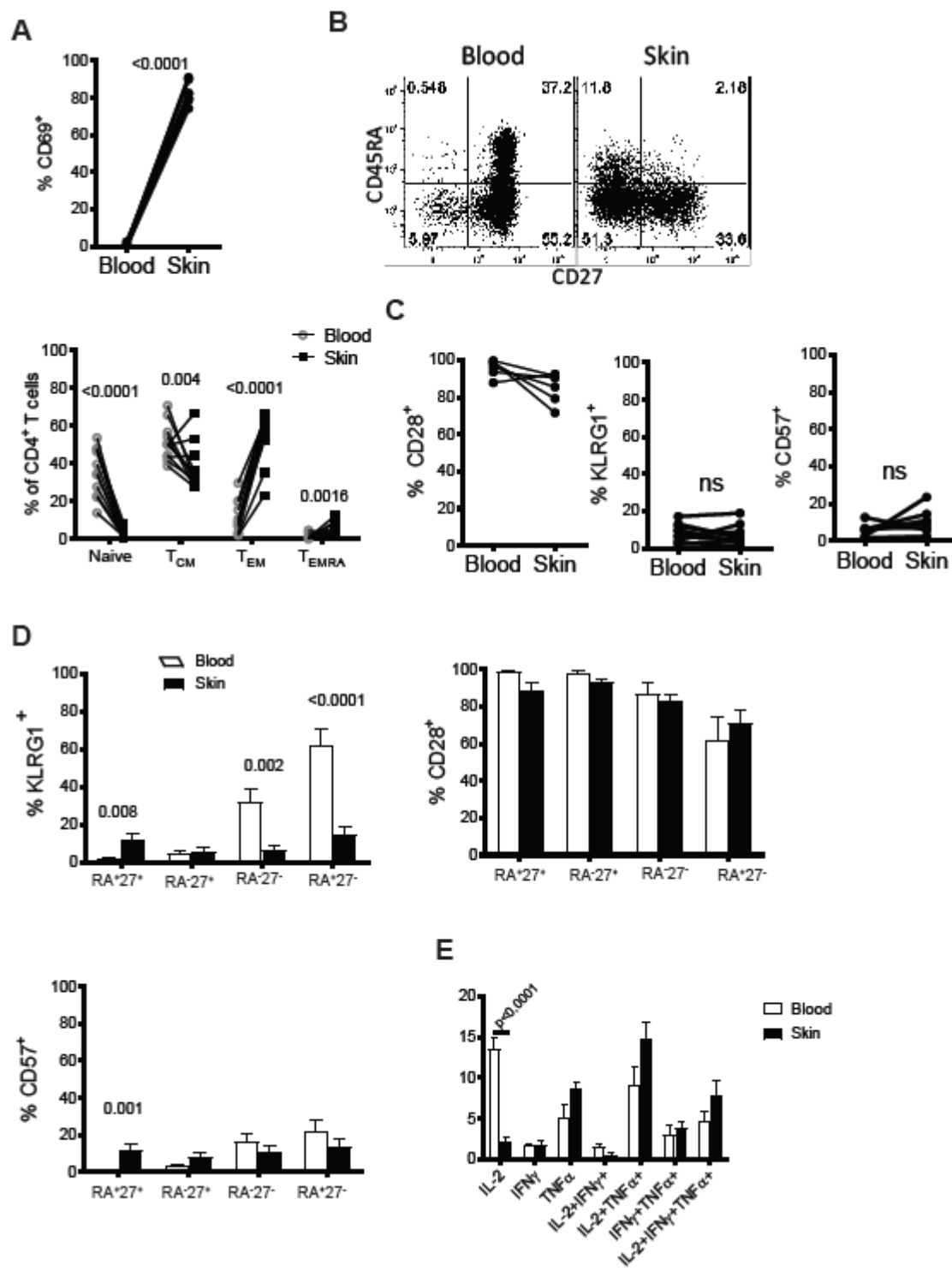


Figure S2.

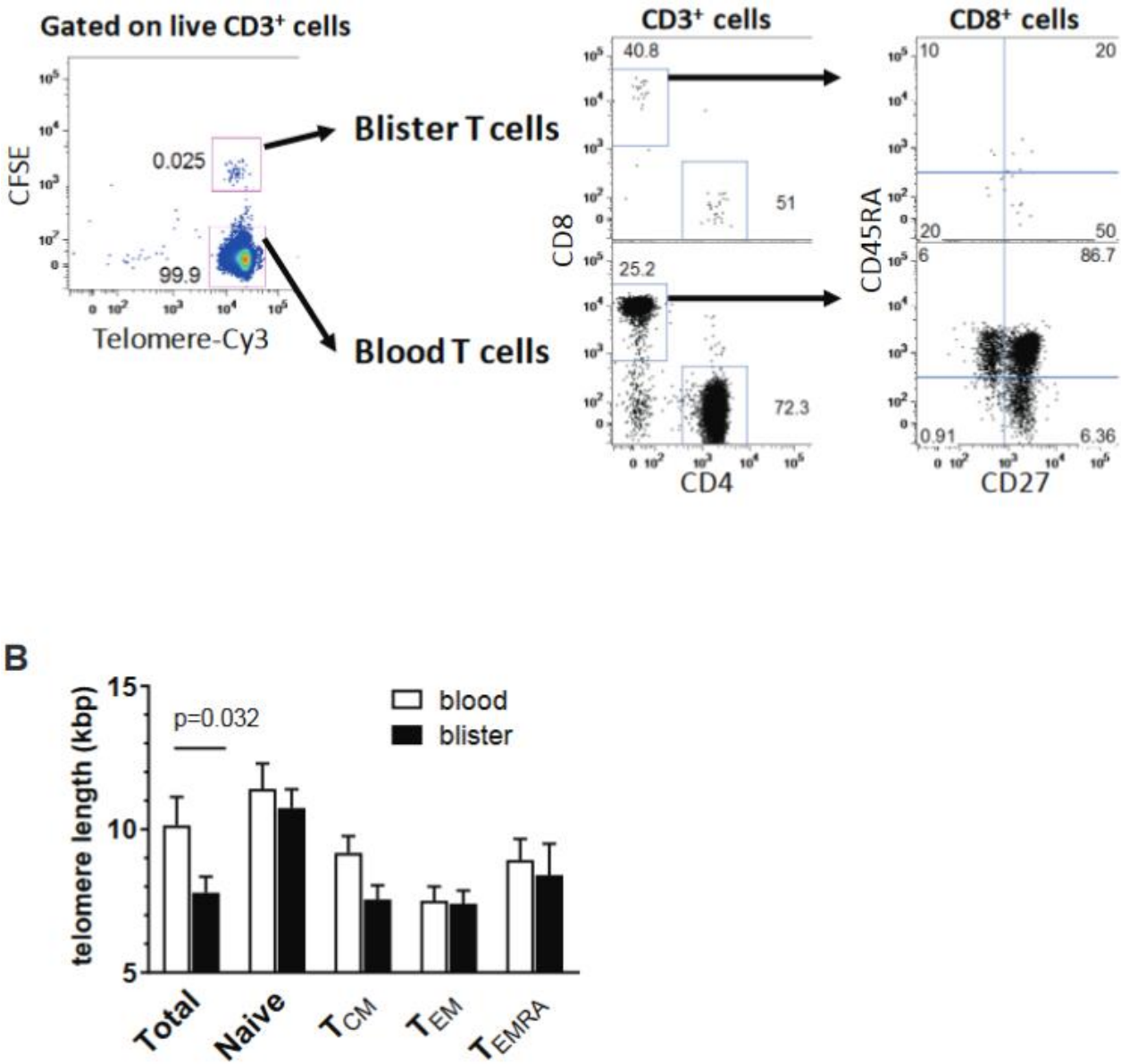


Table S1.

	Target	Clone	Fluorochrome	Manu-facturer
Extracellular targets	CD4	RPA-T4	APC-H7, BrilliantViolet421	BD
		SK3	PE-Cy7	BD
	CD8	SK1	PerCP	BD
		RPA-T8	Biotin	BD
	CD27	0323	APC	eBioscience
		M-T271	FITC ,PE, V500	BD
		L128	AlexaFluor786	BD
	CD25	M-A251	PE-Cy7	BD
	CD28	CD28.2	PE	BD
	CD45RA	HI100	PE-Cy7	BD
		M _{EM} -56	APC	Invitrogen
		HI100	BV605	BD
	CD57	HCD57	APC	Biolegend
	CD69	FN50	FITC	BD
	PD-1	EH12.2H7	PE-Cy7	Biolegend
	CD3	UCHT1	ECD	Beckman Coulter
		SK7	FITC	FITC
	KLRG1	13F12	PE	(A kind gift from H.P. Pircher).
Cytoplasmic targets	IL-2	MQ1-17H12	FITC	BD
	IFN γ	B27	V450	BD
	TNF α	MAb11	APC	BD
	Perforin	δ G9	FITC	BD

	Granzyme B	GB11	AlexaFluor700	BD
Nuclear Targets	Eomes	WD1928	PE-texas red	E-Bioscience
	Tbet	4B10	PE	BD